REMARKS

Applicants respectfully request reconsideration of the present application in view of the foregoing amendments and following commentary. With entry of the present amendment, claim 1 will be revised and pending.

Applicants emphasize how well known it was, in the relevant art, that IL-8 and TNF are effective regulators of the immune system, and that TNF induces IL-8 production. (For instance, see the accompanying article of Schröder *et al.*, *J. Immunology* 144: 2223 (March, 1990), especially at page 2223, lines 28-33.) In light of this fact, a restriction requirement vis-à-vis the recitations of the present claim is improper. Moreover, the Examiner has already acknowledged that the specification is enabled for "the induction of TNF and IL-8 [*sic*]."

Drawings

Applicants concurrently file a formal version of Figure 1. Acceptance of this drawing is respectfully requested in the next communication from the Examiner.

Specification

As requested by the Examiner, the specification had been amended to only refer to the actual drawing and remove all references to the missing drawings, thereby overcoming this rejection. The present specification describes that subject matter of the missing drawings, and therefore the omission of Figures 1-3 has no material effect on the present claims. With respect to Figure 1, the relative location and size of the deletion mutants of p43 are described in detail at page 7 of the specification. For Figure 2, the results of SDS-PAGE analysis of the deletion-mutants of the p43 protein and the p43 protein after purification are described in Examples 1, 2 and 3. The amount of TNF produced, as described in Figure 3, is found at page 15 of the specification.

Priority Document

The Office Action Summary fails to acknowledge applicants' claim for foreign priority under 35 USC 119 and receipt of the priority document. A certified copy of the

priority document Korean Patent Application No. 2001-31310 was filed in parent Application No. 09/930169. Applicants filed a copy of this claim for priority in the present application on April 14, 2004. Acknowledgement of receipt of the certified copy of the priority document is respectfully requested in the next communication from the Examiner.

Information Disclosure Statement

The Examiner has signed and dated the Form SB-08 submitted with the IDS filed on April 14, 2004. The Examiner, however, has not initialed reference A13, cited on the Form SB-08. Accordingly, the Examiner is respectfully requested to return a copy of the SB-08 with reference A13 initialed with the next communication.

Claim Rejections – 35 USC 112, Enablement

Claim 1 is rejected for an alleged lack of enablement. Applicants contend that the rationale for this rejection is inapposite to present claim 1, which the present specification amply enables. A cell THP-1 used the working examples illustrates a cell producing cytokines such as TNF and IL-8, and cells producing TNF and IL-8 were well know at the time of filing. See Cassatella *et al.*, *J. Immunology* 148: 3216 (May, 1992), especially at page 3216, lines 31-34 of the left column and lines 1-5 of the right column.

In further support of this point, applicants present the accompanying experimental data, *in vivo* results with the p43 protein, bearing on induction of macrophage production. The polypeptide of the present invention is the N-terminal sequence of p43. In common with p43, the inventive polypeptide induces production of TNF and IL-8. See the present specification, page 15, and YG *et al.*, *J. Biol. Chem.* 276: 23028 (June, 2001), which is A13 of the Form SB-08, submitted with the IDS filed on April 14, 2004. As YG *et al.* state at page 23030, the cytokine function of p43 is substantially the same as the N-terminal polypeptide of p43 of the present invention, and its activity is superior to EMAP II as the C-terminal of p43.

Therefore, applicants maintain that claim 1 is enabled and the outstanding rejection should be withdrawn.

Rejection for Obviousness-Type Double Patenting

A non-final rejection for Application No. 09/930,169 was mailed on January 14, 2004. Applicants did not file a response to this rejection, therefore, Application No. 09/930,169 is abandoned and the rejection for obviousness-type double patenting is improper and should be withdrawn.

Conclusion

Applicants believe that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested. The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicants hereby petition for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

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IL-1α OR TUMOR NECROSIS FACTOR-α STIMULATE RELEASE OF THREE NAP-1/IL-8-RELATED NEUTROPHIL CHEMOTACTIC PROTEINS IN HUMAN DERMAL FIBROBLASTS[1]

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Human dermal fibroblasts in culture secrete three protein-like neutrophil chemotactic factors, when stimulated either with human rIL-1α or IL-1β; not, however, after incubation with LPS. These three fibroblast-derived neutrophil-activating proteins (FINAP) could be purified by subsequently performed reversed phase and size exclusion HPLC. By high resolution SDS-PAGE, all the proteins were shown to migrate with an M_r of 6,700 (α-FINAP), 3,600 (β-FINAP), and 5,300 (γ-FINAP). All purified cytokine preparations were found to be chemotactic for human neutrophils. In addition, all FINAP induced release of lysosomal enzymes in neutrophils. Deactivation of chemotaxin-elicitable enzyme release showed cross-desensitization of all FINAP with NAP-1/IL-8. Western blot analysis of α-FINAP by using mAb against neutrophil-activating protein (NAP)-1/IL-8 reveals immunologic cross-reactivity with NAP-1/IL-8. By amino-terminal amino acid sequence analysis α-FINAP could be identified as the 77-residue extended form of NAP-1/IL-8 containing the 79-residue form as a minor contaminant. Whereas β-FINAP has been found to be a truncation product of α-FINAP, γ-FINAP shows identity with authentic melanoma growth stimulatory activity with respect to retention time upon reversed phase HPLC, high resolution SDS-PAGE, and biologic properties, as well as amino-terminal amino acid sequence. These data show that human dermal fibroblasts may actively participate in inflammatory reactions by secretion of proinflammatory cytokines.

Intradermal injection of hrIL-1α,[3] hrIL-1β, or hrTNF-α in animals (1, 2) or in humans (3) was recently shown to be followed by a marked tissue accumulation of predominantly PMNL. This infiltration of PMNL after IL-1 injection has originally been suggested to be due to the PMNL chemotactic activity of IL-1 preparations observed in vitro. The finding, however, that hrIL-1β as well as hrTNF-α in vitro are not chemotactic for human PMNL (4, 5) stimulated search for a NAP present as a contaminant in partially purified IL-1 preparations. As a result four laboratories were independently able to isolate and characterize biologically and biochemically a novel cytokine termed MONAP (6), MDNCF (7), NAF (8), LYNAP (9, 10), and chemotactic monokine (11) or NAP-1 which recently was proposed to be termed IL-8 (12). The ability of monocytes (6–8, 11), T lymphocyte preparations (9, 10) as well as cultivated umbilical vein endothelial cells (13) to secrete NAP-1/IL-8 or related proteins raises the question whether dermal fibroblasts may similarly contribute to the accumulation of PMNL after intradermal injection of IL-1.

Data from a recent report indicate that human dermal fibroblasts indeed are able to produce a heat stable, protein-like chemotaxin (14). However, to our knowledge this factor was not further characterized biochemically as well as functionally. In reports by others a PMNL-stimulating factor has been detected in IL-1-stimulated human synovial cell cultures predominantly consisting of fibroblasts. Partial purification of this factor revealed two cytokines with an apparent M_r of 13,000 and 6,000, respectively, as shown by size exclusion HPLC (15, 16).

Furthermore, Northern blot analysis indicated that dermal fibroblasts express NAP-1/IL-8 mRNA when stimulated with IL-1 or TNF (17–19). By nonradioactive in situ hybridization as well as by use of mAb we recently confirmed the expression of NAP-1/IL-8 mRNA as well as secretion of NAP-1/IL-8-like immunoreactivity (20).

In this report, purification and biologic and biochemical characterization of human dermal FINAP will be presented. As a result we were able to identify three FINAP. By amino terminal amino acid sequencing of these proteins the predominant and most potent one proved to represent an extended form of NAP-1/IL-8.

MATERIALS AND METHODS

Fibroblast culture. Human surgically removed skin was incubated with a mixture of 0.05% (w/v) trypsin and 0.02% (w/v) EDTA over night at 4°C, and epidermis was removed thereafter. Dermis was cut into pieces of about 2 mm in diameter. These were transferred into 25-mm² flasks and supplied with RPMI 1640 (GIBCO-BRL, Eggenstein, FRG), supplemented with penicillin (10 U/ml), streptomycin (100 μg/ml), glutamin (2 mM), and 10% FCS. When dermal fibroblasts had started spreading growth, dermal pieces were removed. Medium was changed once a week. Passage was performed by the use of a mixture of 0.05% (w/v) trypsin and 0.02% EDTA for

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The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported by the Deutsche Forschungsgemeinschaft (Schr 305, 1–2).
[2] Address correspondence and reprint requests to: J.-M. Schröder, Ph.D., Department of Dermatology, University of Kiel, Schittenhelmstr. 7, D 2300 Kiel, Federal Republic of Germany.
[3] Abbreviations used in this paper: hIL-1 and hTNF, human IL-1 and TNF; FINAP, fibroblast-derived neutrophil-activating protein; GRO, the oncogen gro (37); MGSA, melanoma growth-stimulating activity, identical with gro; MPO, myeloperoxidase; NAP-1, neutrophil-activating protein (identical with IL-8), previously termed MONAP, MDNCF, NAF, LYNAP, and GCP; RP, reversed phase; TFA, trifluoroacetic acid; PMNL, polymorphonuclear leukocyte; LTB, leukotriene B.

5 min at 37°C. Fibroblasts from cultures of the 5th to 10th passages were transferred either into 6 well plates (9 cm^2/well), or for preparative purposes into 175-cm^2 flasks (Nunc, Wiesbaden, FRG) and were used at confluence.

Stimulation of cultivated fibroblasts. Confluent growing fibroblasts were stimulated either with hrIL-1α (kindly provided by Hoffmann-La Roche, Basle, Switzerland), hrTNF-α (kindly provided by Dr. Schlick, Knoll/BASF, Ludwigshafen, FRG) or LPS (*Salmonella minnesota*, Calbiochem, Marburg, FRG) in RPMI 1640 supplemented with penicillin, streptomycin, and glutamin in the absence of FCS for 24 h at 37°C. As a control experiment, fibroblasts were incubated with medium in the absence of FCS for the same time. Supernatants were collected thereafter and frozen below −70°C until further use. In some experiments cells were collected, washed two times with PBS, suspended in 0.1% (v/v) TFA in HPLC-grade water, and after twofold freeze-thawing, stored below −70°C until further use.

Neutrophil isolation. PMNL were isolated from human blood as previously described (6). Briefly, after separation of mononuclear cells by Ficoll centrifugation E- and PMNL-rich pellets were mixed with warm gelatin solution (2.5% (w/v) in 0.9% NaCl) and E were allowed to sediment for 30 min at 37°C. PMNL-rich supernatants were collected and contaminating red cells were lysed by 0.85% (w/v) NH_4Cl for 10 min at room temperature. PMNL were washed twice and finally suspended in PBS containing 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.1% (w/v) BSA (Sigma, Munich, FRG). Purity of PMNL was greater than 97%, with more than 92% neutrophils and 5% eosinophils. Viability exceeded 97% by trypan blue dye exclusion.

Chemotaxis assay. Screening for neutrophil chemotactic factors was performed by using a modification of the "endogenous component chemotaxis assay" described by Creamer et al. (21); however, with β-glucuronidase as marker enzyme as recently described (6). Chemotactic activity was expressed either in PMNL equivalents which migrated through the filter within 1 h or as chemotactic index (6). Defined chemotaxins such as 10^{-8} M C5a, prepared as described (22), 10^{-9} M FMLP (Sigma) and 10^{-8} M LTB_4 (Paesel, Frankfurt, FRG) were used for control.

In some experiments, PMNL chemotaxis was assessed by direct microscopical cell counting as described elsewhere (13) by using Cellulosenitrat filters (Sartorius, Göttingen, FRG, pore size: 5 μm) instead of polycarbonate filters and an incubation time of 1.5 h.

Enzyme release. Liberation of primary PMNL granule enzymes was determined as recently described (6). Briefly, PMNL (10^7/ml PBS) were preincubated with cytochalasin B (5 μg/ml, Sigma). Thereafter samples were added at appropriate dilutions and incubated for 30 min. After centrifugation supernatants were incubated for 18 h with 0.01 M *p*-nitrophenyl-β-D-glucuronide (Sigma) in 0.1 M sodium acetate (pH 4). The enzymatic reaction was stopped by 0.4 M glycine buffer (pH 10).

For fast detection of PMNL-enzyme-releasing activity, supernatants of stimulated PMNL were investigated for MPO activity by incubation with 0.01 M *o*-phenylendiamine-dihydrochloride in 0.1 M citrate/phosphate buffer (pH 5) containing 0.001% (v/v) hydrogen peroxide for 10 min at room temperature. Enzymatic reaction was stopped by adding 2 M H_2SO_4. Enzyme-releasing activity was expressed either in OD_{405} (β-glucuronidase), OD_{486} for MPO release or in percentage of the 100% control (Triton X-100-treated cells).

Desensitization experiments. Cross-reactivities of PMNL chemotaxin-dependent enzyme release were studied as recently described (6) by preincubating 10^7 PMNL with different chemotaxins at optimal concentrations for 20 min at 37°C. After adding cytochalasin B (5 μg/ml) and subsequent incubation for 5 min, cells were challenged with different stimuli and incubated for additional 30 min. Subsequently release of β-glucuronidase was determined as described above.

Purification of FINAP. Pool supernatants (500 ml per experiment) of hrIL-1α (100 U/ml)- and hrTNF-α (50 ng/ml)-stimulated confluent growing human dermal fibroblasts (corresponding to 2×10^6 cells) were acidified with TFA to pH 3, concentrated by using an Amicon YM-2 filter, and clarified by centrifugation.

HPLC. All HPLC was performed at room temperature with a Spectra Physics liquid chromatography system equipped with a pump, a SP 8700 solvent delivery system, a Kratos spectroflow 783 spectrophotometer (Kratos, Westwood, NJ), and a SP 4270 integrator.

Preparative wide pore RP-8-HPLC. Concentrated supernatants of stimulated fibroblasts or lysates of stimulated fibroblasts were applied to a preparative wide pore RP-8-HPLC column (300-7 C8 Nucleosil, 250 × 12.6 mm, Macherey und Nagel, Düren, FRG) and proteins eluted from the column using a gradient of increasing concentrations of acetonitrile in 0.1% TFA (flow: 3 ml/min).

During the HPLC separation of proteins, absorbance was monitored at 215 nm. Integration values obtained by the peak integrator were used to determine the amounts of proteins and proteins eluting in a given peak. To convert integration units to protein concentrations known amounts of ubiquitine (Sigma) were used for calibration. Nearly 10^6 integration units corresponded to 1 μg ubiquitine.

Size exclusion HPLC. In some experiments fractions off preparative wide pore RP-8-HPLC containing PMNL stimulating activity were concentrated by partial lyophilization and applied to a TSK-2000-3SW-HPLC column (LKB, 0.8 × 60 cm and precolumn), previously equilibrated with 0.1% TFA in HPLC grade water. Polypeptides were separated by using the same solvent at a flow rate of 1 ml/min. For calibration of M_r ubiquitine (Sigma, M_r: 8,500) as well as aprotinine (Sigma, M_r: 6,400) were used.

CN-propyl-RP-HPLC. Biologic active fractions off either preparative RP-8-HPLC or TSK-2000-HPLC were applied to a wide pore CN-propyl-RP-HPLC column (5 μm, 250 × 4.6 mm, J. T. Baker, Gross Gerau, FRG) previously equilibrated with 0.1% TFA in HPLC grade water. Proteins eluted with a gradient of increasing concentrations of *n*-propanol containing 0.1% TFA in water.

Narrow pore-RP-18-HPLC. Fractions off CN-propyl-RP-HPLC containing PMNL-stimulating activity were directly applied to a narrow pore RP-18-HPLC column (Nucleosil—5 μ-octadecyl-silica (RP-18), without endcapping, 250 × 4.6 mm, Bischoff, Leonberg, FRG) previously equilibrated with 0.1% TFA containing 10% (v/v) HPLC grade acetonitrile. Polypeptides eluted with a gradient of increasing concentrations of acetonitrile containing 0.1% TFA.

SDS-PAGE. SDS-PAGE for polypeptides was performed as described by Schägger and von Jagow (23) by using a discontinous system optimized for detection of 1- to 20-kDa polypeptides. For separation of polypeptides a gel containing 16.5% T and 6% C in the presence of 8 M urea at pH 8.45 was used. Polypeptides were fixed with 30% (v/v) isopropanol and 10% (v/v) glacial acetic acid in water containing 0.1% (v/v) glutaraldehyde and were visualized by the use of a silver staining kit (Sigma).

Western blot analysis. After running SDS-PAGE protein bands were transferred to nitrocellulose in a Sartoblot apparatus (Sartorius, Göttingen, FRG). Anodal filter papers were soaked in 0.3 M Tris/HCl containing 20% methanol (pH 10.4) and 25 mM Tris/HCl, 40 mM ϵ-amino-caproic acid containing 20% methanol (pH 9.4) (24).

The electroblotting was done for 2 h at 2 mA/cm^2 gel. Thereafter nitrocellulose sheets were blocked for 1 h in 1% (w/v) gelatin-PBS and incubated overnight with 10 μg/ml mAb against NAP-1/IL-8 in roller bottles followed by washing in PBS. After reaction with rabbit anti-mouse HRP conjugates (Jackson Immunoresearch Labs.) for 1 h foiles were developed as described elsewhere (25).

Amino acid sequence analysis. Intact proteins were analyzed by using an Applied Biosystem gas phase sequencer model No. 470A with on line HPLC analysis of the phenylthiohydantoin derivatives, model No. 120 A.

Estimation of NAP in culture supernatants. Supernatants of fibroblasts nearly growing to confluence in 6-well plates and stimulated with IL-1α (100 U/ml), TNF-α (50 ng/ml), LPS (1 μg/ml), or RPMI 1640 (volume: 1.5 ml in each experiment) for 24 h were tested for NAP-like activity either by determination of PMNL chemotactic activity or determination of NAP-1/IL-8 immunoreactivity by using an ELISA.

Chemotaxis experiments with supernatants were performed as described in "Chemotaxis Assay" at serial dilutions. The dose necessary to evoke a half-maximal response was determined and the number of half-maximal chemotaxis doses present in the whole supernatant calculated. One ED_{50} for fibroblast-derived NAP-1/IL-8 (α-FINAP) was found to be 6.2 ng/ml (see *Results*) and therefore the amount of biologically active NAP-1/IL-8 was estimated in nanograms per experiment.

For determination of NAP/IL-8-immunoreactivity supernatants of stimulated cells were diluted with coating buffer (0.1 M sodium carbonate buffer, pH 10.5) and applied to a 96-well ELISA plate (Nunc). A direct ELISA was performed as described by using either the mAb 8C4 or the mAb 42E4, both directed against NAP-1/IL-8 (25). For calibration and calculation of the amounts of NAP-1/IL-8 immunoreactivity secreted by fibroblasts known amounts of authentic NAP-1/IL-8 (isolated as described elsewhere) (6), diluted with RPMI 1640, and treated similarly as samples obtained from fibroblasts, were used. Fibroblast-derived NAP-1/IL-8-like activity is expressed in nanograms NAP-1/IL-8 per 10^6 fibroblasts.

RESULTS

Production of NAP by IL-1α- or TNF-α-stimulated fibroblasts. When supernatants of cultivated IL-1α- or TNFα-stimulated dermal fibroblasts were assayed in the Boyden chamber system neutrophil chemotactic activity was constantly observed. After the concentration of supernatants with an Amicon YM-2-filter PMNL-chemotac-

TABLE I

Analysis of PMNL chemotactic activity in the retentate and filtrate of Amicon YM-2-concentrated supernatants of IL-1α-stimulated fibroblasts[a]

Assay	Chemotactic index[b] with Reciprocal Dilution:							
	1	2	4	8	16	32	64	128
Retentate	2.9	3.0	3.4	3.4	3.1	2.9	2.4	1.6
Filtrate	1.3	1.2	1.1	1.2	1.0	1.0	1.0	1.1

[a] The 48-h culture supernatants (1.5 ml) of hrIL-1α-stimulated dermal fibroblasts (5×10^6) were concentrated 10-fold by using Amicon YM-2 filters and both the retentate and filtrate collected. The retentate was diluted to the original volume with RPMI 1640 and both retentate and filtrate tested for PMNL chemotactic activity at serial dilutions with the use of the indirect chemotaxis assay as detailed in *Materials and Methods*.

[b] The chemotactic index represents the quotient of stimulated migration and random migration.

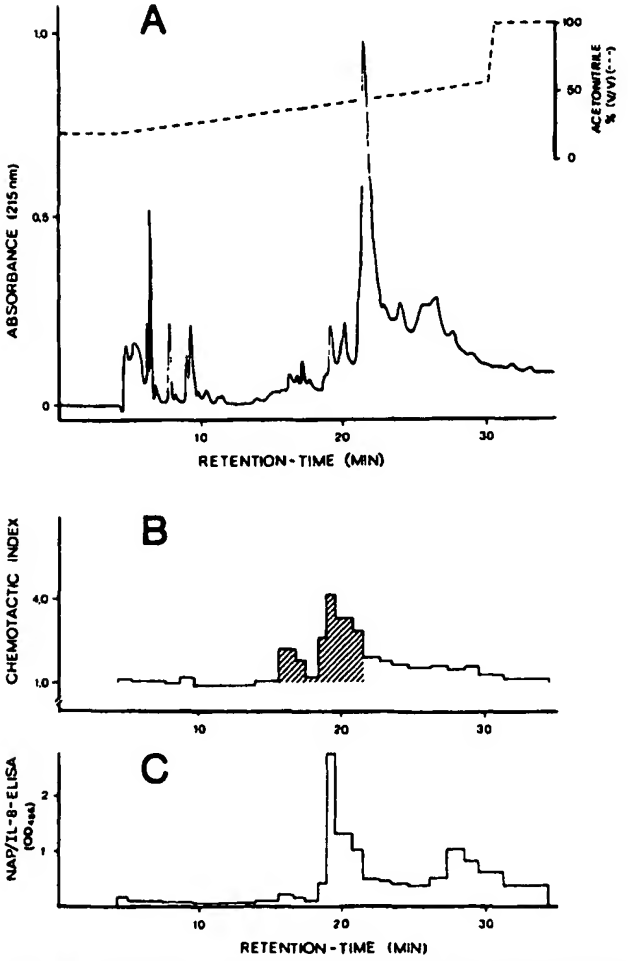

Figure 1. Preparative wide pore RP-8-HPLC of supernatants obtained from hrIL-1α-stimulated fibroblasts. Supernatants of hrIL-1α-stimulated human dermal fibroblasts were acidified to pH 3, concentrated, and applied to a preparative wide pore RP-8-HPLC column. *A*, Chromatogram of the supernatant. *B*, PMNL-chemotactic activity of column effluents. The *hatched area* indicates fractions containing PMNL-MPO-releasing activity. In C, NAP-1/IL-8 immunoreactivity by using the NAP-1/IL-8 mAb 8C4 and a direct ELISA. Note strong NAP-1/IL-8 immunoreactivity near the peak only with biologic activity eluting at 19.6 min. A typical experiment is shown.

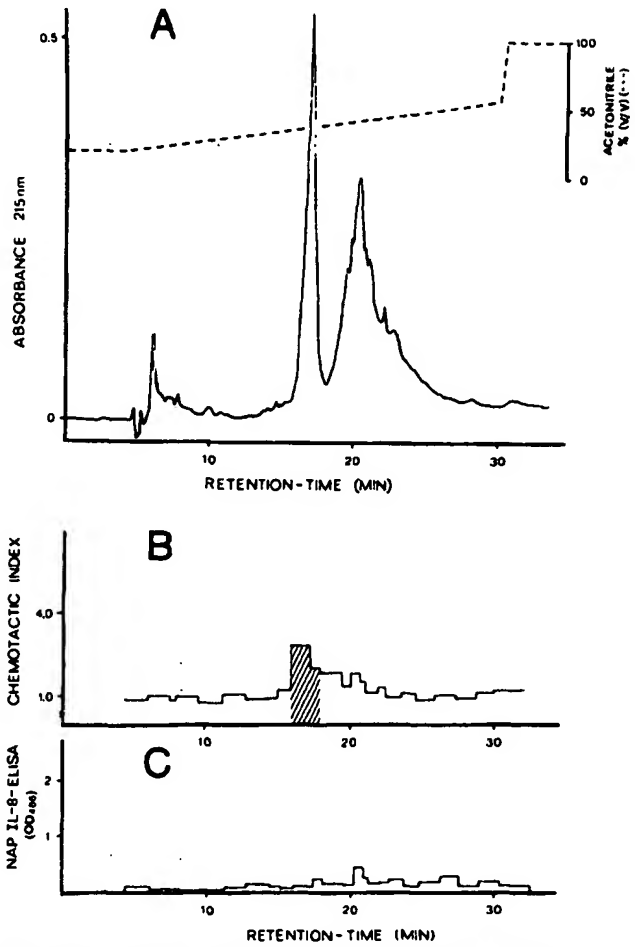

Figure 2. Preparative wide pore RP-8-HPLC of lysates obtained from hrIL-1α-stimulated fibroblasts. hrIL-1α-stimulated dermal fibroblasts were lysed by threefold freeze thawing in 0.1% TFA and after clarification lysates were applied to a preparative wide pore RP-8-HPLC column. *A*, Absorption of the effluent at 215 nm. PMNL chemotactic activity of each fraction was determined (*B*). The *hatched area* indicates fractions containing PMNL-MPO-releasing activity. *C*, NAP-1/IL-8 ELISA-reactivity, which was determined as indicated in Figure 1C. Note the absence of NAP-1/IL-8 immunoreactivity in fractions containing major biologic activity. A typical experiment is shown.

tic activity was investigated in serial dilutions of the retentate as well as filtrate. High titer chemotactic activity exclusively in the retentate was detected (Table I).

Purification of the fibroblast-derived neutrophil activating factor. In previous work analyzing PMNL-activating proteins in supernatants of monocytes (6), T lymphocyte preparations (9) or human umbilical vein endothelial cells (13) we started with a G-75 gel filtration of concentrated supernatants. In the case of fibroblast supernatants, the lower protein content made it possible to start purification of PMNL-activating proteins by a preparative wide pore RP-HPLC step. Figure 1*A* shows the chromatogram of supernatants obtained from IL-1/TNF-stimulated fibroblasts directly applied to this HPLC column. Two peaks of PMNL-chemotactic as well as PMNL-MPO-releasing activity eluted at 17.2 min and 19.6 min (Fig. 1*B*). The latter peak showed strong NAP-1/IL-8

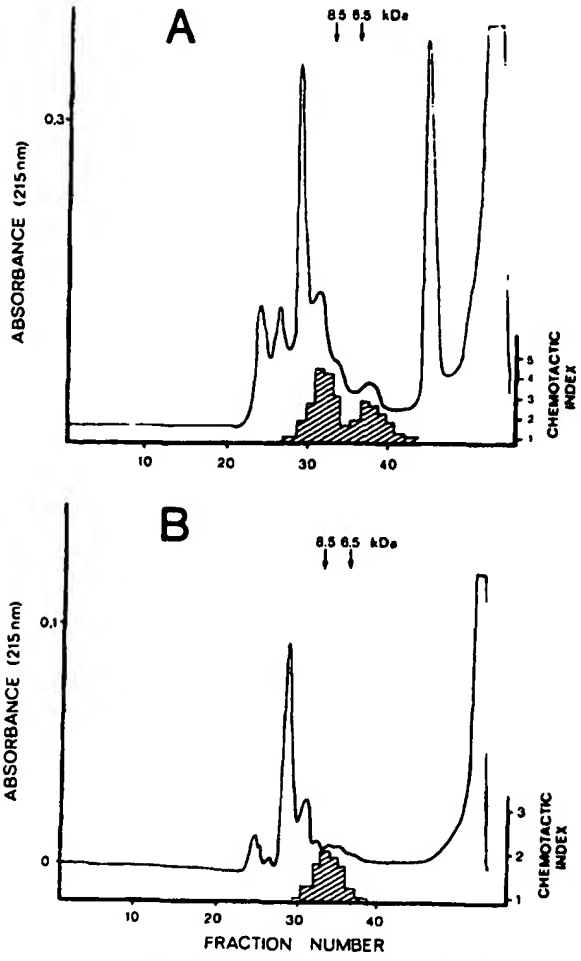

Figure 3. TSK-2000-size exclusion HPLC of fibroblast-derived neutrophil chemotactic factors. *A* TSK-2000-HPLC profile of the major chemotaxin obtained from supernatants of IL-1α-stimulated dermal fibroblasts off preparative RP-8-HPLC. Note the presence of two peaks with biological activity. *B*, TSK-2000-HPLC-profile of the major chemotaxin obtained from lysates of hrIL-1α-stimulated fibroblasts. *Arrows* indicate the elution position of ubiquitine (8.5 kDa) and aprotinine (6.5 kDa) for estimation of the M_r (×1000).

ELISA-reactivity by using all four of our mAb (Fig. 1*C* and data not shown). When lysates of IL-1/TNF-stimulated fibroblasts were chromatographed on the same column (Fig. 2*A*), a single fraction eluted at 16.8 min containing both PMNL-chemotactic- as well as PMNL-MPO-releasing activity (Fig. 2*B*). This was found to be identical with the 17.2 min eluting chemotactic factor in supernatants. In contrast to the factor eluting at 19.6 min present in supernatants of stimulated fibroblasts, this fraction did not show immunoreactivity in the NAP-1/IL-8 ELISA system (Fig. 2C).

For SDS-PAGE-analysis and biologic characterization, all of the NAP present in supernatants as well as in fibroblast lysates were purified further. Figure 3 shows the TSK-2000-size exclusion HPLC-profile of the major supernatant PMNL-chemotaxin (Fig. 3*A*) as well as the lysate-PMNL chemotaxin (Fig. 3*B*).

It appears that the major supernatant chemotaxin consists of two neutrophil chemotactic factors, one eluting at 15 min, which corresponds to a M_r of nearly 11,000 (α-FINAP) and a second one of either lower amount or lower potency with a M_r near 6,000 (β-FINAP) (Fig. 3*A*). Another PMNL chemotactic factor (γ-FINAP) present in supernatants as well as lysates of IL-1-stimulated fibroblasts eluted as a single peak with a M_r near 10,000.

CN-propyl-RP-HPLC of preparative RP-8-HPLC purified PMNL chemotactic protein α-FINAP is shown in Figure 4*A*, whereas Figure 4*B* shows the HPLC profile of γ-FINAP. In both cases, PMNL-MPO-releasing activity eluted in the early effluent. Final purification of PMNL-stimulating proteins in supernatants as well as lysates of stimulated fibroblasts was assessed by the use of narrow pore RP-18-HPLC. Figure 5*A* shows the profile of the major NAP α-FINAP (M_r ~ 11,000) present in fibroblast supernatants. The amount of this PMNL-chemotaxin collected from four 175-cm² flasks containing nearly confluent growing fibroblasts (which corresponds to nearly 10^8 cells) was calculated to be near 13.5 μg.

The 6-kDa PMNL-activating protein β-FINAP could be separated from α-FINAP only when TSK-2000-size exclusion HPLC of the RP-8-HPLC-purified supernatant PMNL-chemotactic factor was performed. Final purification was achieved by using CN-propyl-HPLC (data not shown) followed by narrow pore RP-18-HPLC (Fig. 5*B*). The retention time of this β-FINAP is similar to that of the major 11-kDa chemotaxin (α-FINAP). When the TSK-2000-HPLC-step was omitted, a mixture containing α- and β-FINAP was obtained. In addition, when supernatants of IL-1-stimulated fibroblasts were passed over an anti-NAP-1/IL-8-affinity column prepared with our mAb and bound proteins were analyzed by RP-HPLC, a mix-

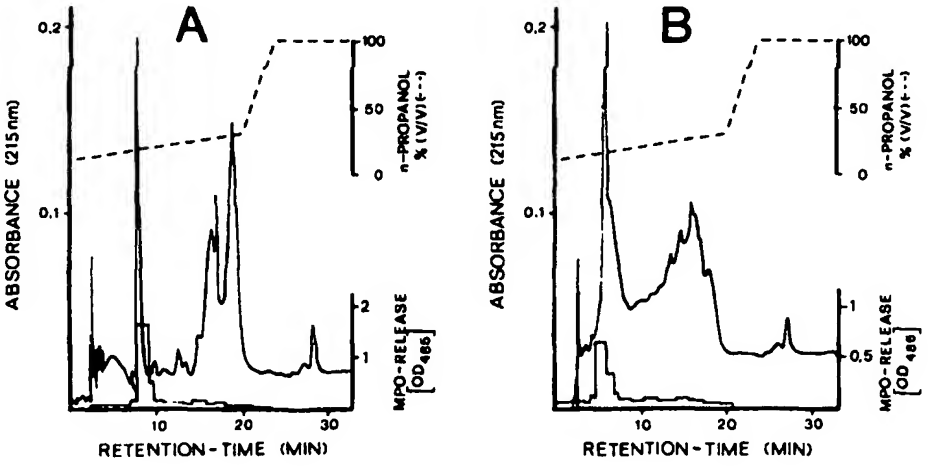

Figure 4. CN-propyl-RP-HPLC of preparative RP-8-HPLC-purified fibroblast-derived neutrophil chemotactic proteins. *A*, profile of the major chemotaxin (α-FINAP) present in supernatants of IL-1-stimulated fibroblasts. The *shaded area* shows the fraction containing PMNL-MPO-releasing activity. *B*, CN-propyl-RP-HPLC profile of the major PMNL-chemotaxin (γ-FINAP) in lysates of IL-1-stimulated fibroblasts. The *shaded area* indicates MPO-releasing activity.

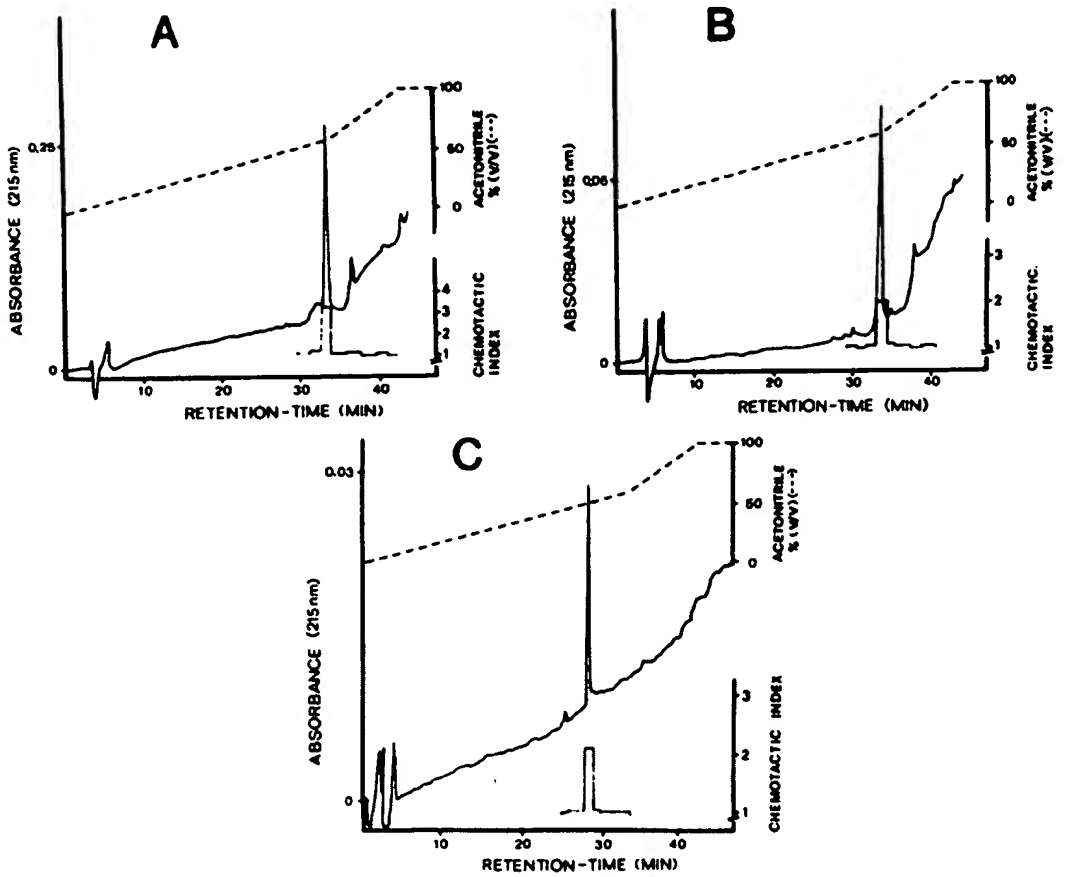

Figure 5. Final RP-18-HPLC purification of FINAP. *A*, Narrow pore RP-18-HPLC-profile of an α-FINAP-preparation purified by RP-8-HPLC, TSK-2000-HPLC, and CN-propyl-RP-HPLC. Only the major peak absorbing at 215 nm contains highly potent PMNL-chemotactic activity (shaded area) as well as PMNL-MPO-releasing activity (not shown). *B*, RP-18-HPLC-profile of the low M_r fibroblast-derived neutrophil chemotactic protein (β-FINAP). A preparation prepurified by RP-8-HPLC, TSK-2000-HPLC, and CN-propyl-HPLC was applied to the RP-18-HPLC column. Only the major at 215-nm absorbing peak contained chemotactic activity (*shaded area*). Final purification of γ-FINAP (the major fibroblast-lysate derived chemotactic factor) by RP-18-HPLC is shown in *C*. The peak eluting at 28.5 min showed PMNL chemotactic activity (*shaded area*).

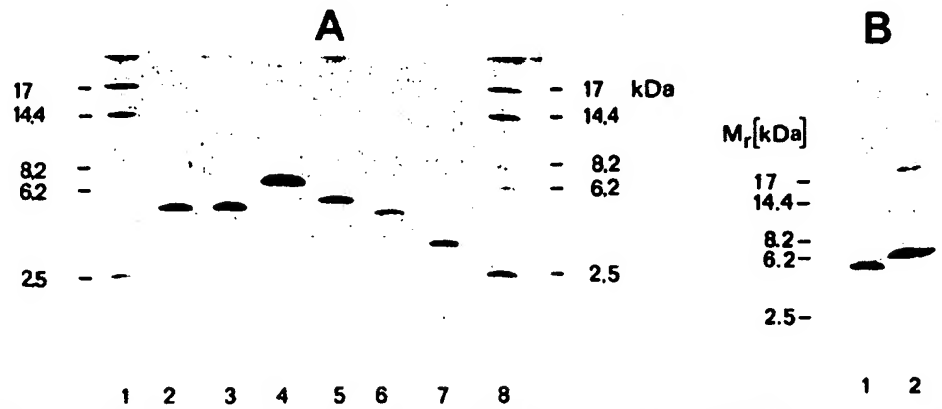

Figure 6. SDS-PAGE of fibroblast-derived neutrophil chemotactic proteins. Picture A shows the SDS-PAGE profile of RP-18-HPLC-purified PMNL chemotactic proteins under nonreducing conditions as detailed in *Materials and Methods*. For estimation of the M_r in *lanes 1* and *8*, Br-CN-cleavage products of myoglobin were applied. *Lane 2* contains γ-FINAP (50 ng), *lane 3* contains 50 ng of MGSA obtained from psoriatic scales (35), whereas *lane 4* was loaded with 80 ng of α-FINAP. *Lane 7* was loaded with 40 ng of β-FINAP. For direct comparison *lane 5* contained 60 ng authentic NAP-1/IL-8, whereas *lane 6* was loaded with 30 ng of authentic endothelial cell-derived NAP (13). Polypeptides were stained with silver. Figure 6*B* shows the Western blot experiment by using 8C4 mAb against NAP-1/IL-8. *Lane 2* was loaded with an α-FINAP preparation (80 ng), whereas *lane 1* contained authentic NAP-1/IL-8 (70 ng). Note the presence of an additional faint band near 25 kDa in the α-FINAP preparation, which is also detectable by Western blot.

ture of α- and β-FINAP was obtained as assessed by SDS-PAGE analysis (data not shown). Final purification of γ-FINAP, which is present in supernatants as well as lysates of IL-1-stimulated fibroblasts, by RP-18-HPLC (Fig. 5C) revealed a single peak absorbing at 215 nm, eluting several minutes earlier than both NAP α-FINAP and β-FINAP.

High resolution SDS-PAGE of these three FINAP under nonreducing condition is shown in Figure 6*A*. In addition, authentic NAP-1/IL-8 showing a mobility like a 5.6-kDa-

TABLE II

Chemotactic activity of α-, β-, and γ-FINAP by using a direct cell counting method[a]

Stimulus	Cells/High Power Field[b]
α-FINAP (30 ng/ml)	128 ± 34
β-FINAP (300 ng/ml)	112 ± 28
γ-FINAP (10 ng/ml)	68 ± 14
NAP-1/IL-8 (5 ng/ml)	134 ± 21
Buffer	23 ± 4

[a] Blind well Boyden chambers were filled with the appropriate stimulus in PBS containing 0.1% BSA. A cellulose nitrate filter (pore size 5 μm) covered the chemoattractant-containing compartment. PMNL (2×10^5) were used in each experiment. After incubation at 37°C, filters were fixed, stained, and mounted on glass slides and migrated cells were counted under a microscope at five randomly distributed high power fields.

[b] Results are the mean of two triplicate experiments ± SD.

polypeptide is applied in *lane 5*, as well as a mixture of myoglobulin fragments with known m.w. in *lane 1* and *8* for calibration.

α-FINAP migrates as a 6.7-kDa polypeptide with a sometimes faint band at 5.6 kDa, β-FINAP as a 3.6-kDa polypeptide, whereas the lysate derived PMNL-activating protein γ-FINAP shows a mobility of a 5.3-kDa polypeptide. Figure 6*B* shows the Western blot experiment with an α-FINAP-preparation giving one band at 6.7 kDa and a second (faint) band of a 25-kDa protein, which is detectable in that α-FINAP preparation also by silver staining (Fig. 6*A*). Similar results were obtained with our three other mAb against NAP-1/IL-8 (data not shown).

Biologic characterization of FINAP. All FINAP preparations were found to be chemotactic for PMNL when tested in the Boyden chamber system (Table II). It appears that both α- and β-FINAP are potent chemotaxins by the number of migrated cells as a parameter.

These results were substantiated by the use of an indirect cell-counting chemotaxis assay. As shown in Figure 7 among the three cytokines α-FINAP is the most potent fibroblast-derived PMNL chemotaxin as shown by the number of migrating cells at optimal stimulation conditions as well as by determinating the ED_{50}, which was calculated to be near 6.2 ng/ml. At optimal concentrations an β-FINAP preparation induces migration of similar numbers of cells, however the ED_{50} is 50-fold higher than that seen for α-FINAP (Fig. 7). γ-FINAP shows a similar ED_{50} as obtained with α-FINAP; however, its dose response curve is bell-shaped covering a more narrow area. The number of migrated cells at optimal concentration is only 80% of that seen for α-FINAP (Fig. 7).

Although in addition to stimulation of chemotactic migration all FINAP induce a dose-dependent release of β-glucuronidase in cytochalasin B pretreated PMNL, β-FINAP has been found to be a very weak inducer of enzyme release (Fig. 8). Cross-desensitization experiments with α-FINAP, β-FINAP, γ-FINAP, and NAP-1/IL-8 revealed cross-desensitization with each other; not, however, with C5a (Table III). Moreover, when PMNL were preincubated with α-, β-, or γ-FINAP, enzyme release after a second challenge with NAP-1/IL-8 is drastically (more than 80%) reduced only with α-FINAP. β-FINAP as well as γ-FINAP showed a weaker inhibition under these conditions (Table III).

Amino-terminal sequencing of FINAP. α-FINAP could be purified to apparent homogeneity in sufficient amounts for amino-terminal amino acid sequence analysis. The sequence obtained for the first 10 amino acids was AVLPRSAKEL..., with the single letter code for amino acids (Table IV). A minor component (~15%) read EGAVLPRSAK....

Amino-terminal sequencing of a β-FINAP preparation gave the same sequence as obtained for α-FINAP (Table IV). The sequence obtained for a γ-FINAP preparation read ASVATELRXQXLQT, which is identical to that published for MGSA and GRO (Table IV).

Production of FINAP after stimulation of fibroblasts. Stimulation of cultivated fibroblasts with IL-1α and TNF-α resulted in production of large amounts of FINAP activity, either tested by bioassay or NAP-1/IL-8-ELISA (Table V). LPS failed to stimulate fibroblasts to produce protein-like PMN chemotaxins or NAP-1/IL-8-like immunoreactivity (Table V).

DISCUSSION

Our results show that IL-1α, as well as TNF-α (not however LPS), stimulate human dermal fibroblasts to

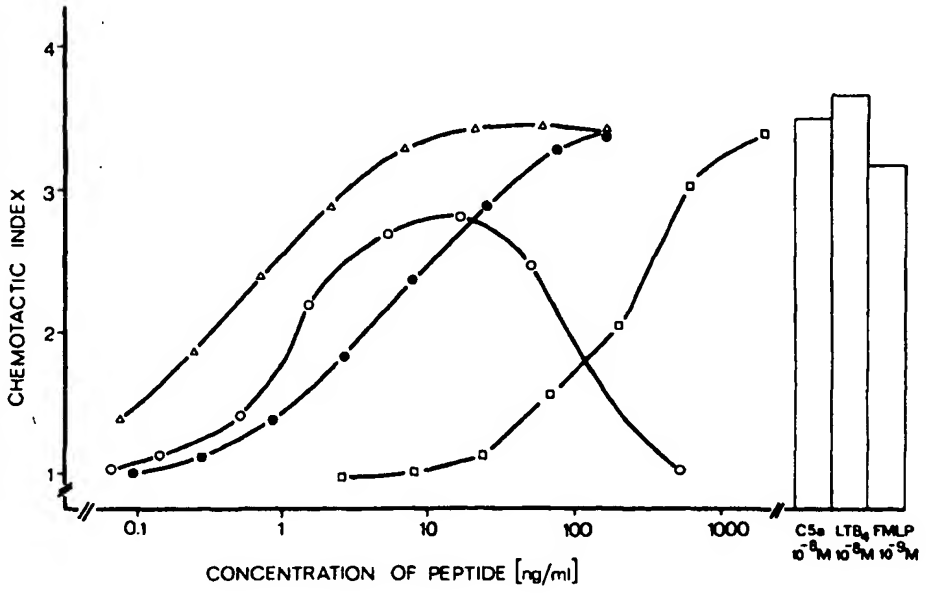

Figure 7. PMNL chemotactic activity of FINAP. PMNL-chemotactic activities of purified preparations of α- (●—●), β- (□—□), and γ-FINAP (○—○) as a function of the protein concentration used are shown. In addition the dose response curve of authentic NAP-1/IL-8 (Δ—Δ) as well as chemotactic indices of well characterized PMNL chemotactic factors like C5a, LTB_4, and FMLP are indicated (as *bars*). Experimental conditions are detailed in *Materials and Methods*. The indirect cell counting method was used for estimation of chemotactic indices. Results are expressed as the mean of three duplicate performed experiments.

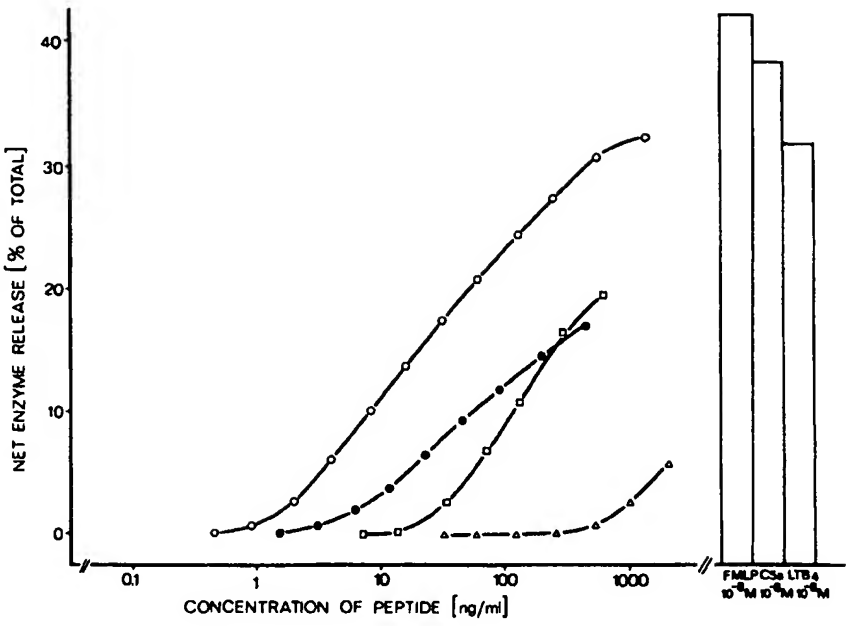

Figure 8. PMNL degranulation-eliciting activity of FINAP. PMNL degranulation (β-glucuronidase)-eliciting activities of purified preparations of α- (□—□), β- (Δ—Δ) and γ-FINAP (●—●) as a function of the protein concentration used are shown. In addition the dose response of authentic NAP-1/IL-8 (○—○) as well as enzyme release by well characterized chemotactic factors such as C5a, FMLP, and LTB_4 (*bars*) are shown. Enzyme release was performed with cytochalasin B-pretreated PMNL as detailed in *Materials and Methods*. The mean of three experiments is shown.

TABLE III

Deactivation of chemotaxin-elicitable PMNL enzyme release by FINAP and known chemotaxins[a]

Preincubation with	Stimulation with				
	C5a (50 ng/ml)	α-FINAP (100 ng/ml)	β-FINAP (200 ng/ml)	γ-FINAP (150 ng/ml)	NAP-1/IL-8 (60 ng/ml)
C5a (50 ng/ml)	21	98	91	106	92
α-FINAP (240 ng/ml)	96	9	0	8	11
β-FINAP (210 ng/ml)	103	54	14	41	74
γ-FINAP (180 ng/ml)	95	36	0	7	31
NAP-1/IL-8 (120 ng/ml)	100	2	0	0	0

[a] Enzyme release (β-glucuronidase) was determined in chemotaxin-preincubated PMNL after subsequent stimulation with different chemotaxins, at given concentrations as shown in *Materials and Methods*. Results are expressed in percentage of net enzyme release of the control (buffer-incubated cells). Values represent the mean of three experiments.

TABLE IV

Amino-terminal amino acid sequence of FINAP

α-FINAP:	85% AVLPRSAKELRXQXIKTYSK....
	15% EGAVLPRSAKELRXQXIK....
β-FINAP:	AVLPRSAKELXXQ...
NAP-1/IL-8[a]:	SAKELRCQCIKTYSK....
3-10 C[b]:	MTSKLAVALLAAFLISAALCEGAVLPRSAKELRCQCIKTYSK....
γ-FINAP:	ASVATELRXQXLQT...
MGSA[c]:	ASVATELRXQXLQTLQG...
GRO[d]:	MARAALSAAPSNPRLLRVALLLLLLVAAGRRAAGASVATELRCQCLQTLQG...

[a] Sequence from Refs. 7, 8, 10, and 11.
[b] Amino acid sequence deduced from the 3-10C-cDNA nucleotide sequence (31). Underlined area indicates the sequence of the predicted signal protein.
[c] Sequence from Ref. 35.
[d] Sequence from Ref. 36.

secrete three neutrophil chemotactic proteins which are distinguishable in retention time as shown by size exclusion HPLC and subsequent RP-HPLC. Moreover our data demonstrate that these two cytokines, IL-1α and TNF-α, induce the synthesis of a PMNL chemotaxin of lower potency (with respect to the number of migrating cells at optimal stimulation conditions).

Results similar to ours were recently obtained in cultivated synovial cells, in which the major proportion of cells are fibroblasts. By size exclusion HPLC, two polypeptides with neutrophil stimulating activity were identified with a M_r of 13,000 and 6,000 (15). For PMNL-chemotactic proteins found in supernatants of IL-1-stimulated dermal fibroblasts we estimated a M_r of 11,000 and 5,000 (Fig. 3), whereas the low M_r PMNL chemotactic factor described in our present study is biologically less active compared with the two other factors (Fig. 7). It appears that the synovial cell-derived PMNL chemotactic factors as described by Watson et al. (15) are similar to both dermal fibroblast-derived cytokines described here, although characterization and biochemical comparison of synovial cell-derived PMNL chemotactic proteins is lacking so far.

Whereas production of NAP-1/IL-8 by dermal fibroblasts was expected after the demonstration of NAP-1/IL-8 specific mRNA within these cells (17–20) our observation that the major PMNL chemotactic proteins found in IL-1- or TNF-stimulated fibroblasts is the NH_2-terminal extended 77 residue variant of NAP-1/IL-8 (Table III) was unexpected.

The presence of the extended form of NAP-1/IL-8 in fibroblasts supernatants, which we tentatively termed α-

TABLE V
Effect of different stimuli upon FINAP secretion[a]

Stimulus	Amounts of NAP-1 (ng/10^6 cells) Determined by		
	Bioassay[b]	ELISA[c]	n
None	<2	<1	6
hrIL-1α (100 U/ml)	52 ± 21	86 ± 27	8
hrTNF-α (50 ng/ml)	29 ± 15	85 ± 30	8
LPS (1 μg/ml)	<2	<1	5

[a] Confluent growing fibroblasts (5×10^6) were stimulated with either stimuli for 24 h and NAP-like activity was determined by bioassay or ELISA as detailed in *Materials and Methods*.
[b] The amount of fibroblast-derived NAP was estimated by serial dilution of culture supernatants and determination of the ED_{50} followed by calculation of the amount produced by 10^6 cells.
[c] A direct NAP-1/IL-8 ELISA. Proteins in supernatants of stimulated fibroblasts were coated to an ELISA plate, incubated wtih the mAb 8C4 raised against NAP-1/IL-8 (23), and bound mAb were detected by the use of peroxidase-conjugated secondary antibodies.

FINAP, is supported also by high resolution SDS-PAGE. By using a recently described method for determination of the M_r of polypeptides in the M_r 1,000 to 20,000 area (23), which was shown in our laboratory to be optimal for separating truncation products of NAP-1/IL-8, we were able to calculate a M_r of 6,700 for α-FINAP, which is higher than that seen for NAP-1/IL-8 in this system (Fig. 6). In earlier work (10) we were able to demonstrate that the 77-residue form of NAP-1/IL-8 is present as a 30% contaminant in T lymphocyte-derived NAP-1/IL-8 preparations. Furthermore, in the supernatants from mitogen- or LPS-stimulated human mononuclear cells nearly 10 to 40% of the total NAP-1/IL-8 were found to consist of extended forms including the 79-residue variant of NAP-1/IL-8 (26, 27), which we found as 15% contaminant in α-FINAP preparations. NAP-1/IL-8 known to be a 72-residue polypeptide with a calculated m.w. of 8386 (10, 28) shows in this SDS-PAGE system an increased mobility with a calculated M_r of 5,600 (Fig. 6).

Moreover, α-FINAP proved to be of higher M_r than the endothelial cell derived NAP-1/IL-8 recently purified in our laboratory (13) and which now has been identified as a three amino-terminal amino acids Ser, Ala, and Lys missing truncated form of NAP-1/IL-8 (our unpublished results). In nearly all of our α-FINAP preparations apart from the 79-residue form of NAP-1/IL-8, which could not be separated from the 77-residue form of NAP-1/IL-8 by high resolution SDS-PAGE, a trace contaminant (<2%) is the 72 residues containing NAP-1/IL-8. This is proven by overloaded SDS-PAGE and silver staining giving a faint line exactly at the same position as authentic NAP-1/IL-8 migrates (data not shown). Moreover, with mAb against NAP-1/IL-8 (25) binding sites were detected in Western blot with α-FINAP, which support closed biochemical similarity to NAP-1/IL-8 (Fig. 6*B*). In addition to the 6.7-kDa band a faint line at 25 kDa present in that α-FINAP preparation showed NAP-1/IL-8 immunoreactivity and may reflect rather an aggregated form of α-FINAP than a precursor form.

The biologic activity profile of an α-FINAP preparation is similar to that seen for NAP-1/IL-8 (6, 29). α-FINAP appears to be a potent PMNL chemotactic factor, eliciting degranulation in cytochalasin B pretreated PMNL (Figs. 7 and 8). The calculated ED_{50} for PMNL chemotaxis, however, has been found to be near 6 ng/ml. This is higher than that we have seen for essentially pure 72-residue NAP-1/IL-8, which is near 0.5 to 1 ng/ml, as found in our laboratory (6) as well as in others (28, 29). In addition, for β-glucuronidase release, higher amounts of α-FINAP were necessary to give responses comparable to NAP-1/IL-8.

The reason for the discrepancy in biologic activity between these two closely related proteins remains speculative at the moment. It appears likely that the extended (pro-protein) forms of NAP-1/IL-8 possess a lower specific activity in eliciting PMNL-chemotactic and degranulation-responses as compared with NAP-1/IL-8. However, principally it cannot be excluded that α-FINAP completely lacks PMNL-stimulating activity and that trace amounts of contaminating NAP-1/IL-8, as we have noted to be present in all of our α-FINAP preparations, are responsible for chemotactic activity. Such a suggestion is supported by recent investigations of possible PMNL function-stimulating activity of β-thromboglobulin, a platelet α-granule product which is structurally related to NAP-1/IL-8.

Whereas β-thromboglobulin has been shown to lack PMN chemotactic activity, as found also for its precursor forms of the connective tissue-activating protein III, CTAP III, as well as platelet basic protein (26), a cleavage product of β-thromboglobulin missing 11 amino acids at the NH_2 terminus has been detected to be a potent neutrophil activator (30).

So far, we were unable to separate these traces of highly active NAP-1/IL-8 from the extended form of NAP-1/IL-8. Difficulties in separating extended forms from native NAP-1/IL-8 have recently been reported (27). From that work it appears that these pro-proteins may undergo enzymic degradation toward NAP-1/IL-8, which makes separation quite difficult (27).

It is interesting to note that fibroblasts nearly exclusively produce both the 77- and 79-residue extended forms of NAP-1/IL-8, whereas mononuclear cells secrete NAP-1/IL-8 as the predominant form (10, 27, 28). This points toward different peptidase activities present in these two cell types as being responsible for amino-terminal truncation of the NAP-1/IL-8 precursor form, as deduced from the nucleotide sequence of 3-10C-cDNA (31).

The second PMNL chemotactic protein secreted by dermal fibroblasts that we tentatively termed β-FINAP is of low M_r. The amounts necessary for half-maximal stimulation of chemotaxis are 50-fold higher than those needed for NAP-1/IL-8. It appears that this factor is a weak agonist compared with NAP-1/IL-8. Binding of β-FINAP to the same receptor on human neutrophils is likely—although not proven—by desensitization of PMNL biologic responses with NAP-1/IL-8. It is not likely, however, with C5a, which is known to bind to a different PMNL receptor (32). A separate NAP-1/IL-8 receptor on neutrophils has been postulated (6, 29) and was recently identified (33).

Also in the case of β-FINAP we cannot exclude a trace contamination by α-FINAP. Therefore it is entirely possible that some of the chemotactic activity of β-FINAP preparations may derive from a trace of highly active NAP-1/IL-8. Nevertheless, it appears that β-FINAP is a C-terminal truncation product of α-FINAP. This is proven by amino-terminal amino acid sequence analysis (Table IV) as well as trapping experiments with anti-NAP-1/IL-

8 affinity columns. A similar polypeptide with low M_r we also detected in NAP-1/IL-8 preparations isolated from purified monocytes (25, 28). So far, that truncation product was not extensively studied, however when directly compared in SDS-PAGE it showed a M_r of 3,000, which is slightly lower than that seen for β-FINAP (Fig. 6). This may support the suggestion that there do exist also C-terminal truncation products of NAP-1/IL-8 apart from NH_2-terminal variants.

Interestingly, an intracellular PMNL-chemotactic protein was discovered within IL-1α- or TNF-α-stimulated fibroblasts. A similar amount of the same chemotaxin is also detectable in supernatants of stimulated cells and could clearly be separated from the two other NAP-1/IL-8-related proteins by RP-18-HPLC (Fig. 5). So far, we were able to purify this chemotaxin tentatively named γ-FINAP to apparent homogeneity. This chemotactic factor appears to be related to NAP-1/IL-8 because in the deactivation assay γ-FINAP shows cross-desensitization with NAP-1/IL-8; not, however, with C5a (Table III). This is further proven by an NH_2-terminal amino acid sequence analysis. γ-FINAP shows a sequence identical to that published for the melanoma growth-stimulating activity factor MGSA (35), known to be the gene product of the oncogen GRO (36). The dose response curve of PMNL chemotactic migration shows a similar ED_{50} as that seen for NAP-1/IL-8 (Fig. 7). In contrast to this, however, γ-FINAP appears to be of lower potency, inducing smaller numbers of PMNL to migrate upon optimal stimulation.

Moreover, we recently purified a protein with identical biologic activities (ED_{50} of PMNL-chemotaxis, cross-desensitization with NAP-1/IL-8, profile of the dose response curve) from extracts of psoriatic scales (34). This factor was analyzed for its amino-terminal amino acid sequence (34) and it was shown that this chemotaxin is identical to MGSA (36). Indeed, γ-FINAP and the psoriasis-derived MGSA were identical by retention time upon RP-HPLC (data not shown) as well as high resolution SDS-PAGE (Fig. 6).

MGSA/GRO possesses 56% structure homology with NAP-1/IL-8 containing four cysteines at the same position (35). So it is possible that MGSA/GRO is a (weaker) agonist binding to the NAP-1/IL-8 receptor on neutrophils although this needs further clarification.

It is well documented that fibroblast cell lines, including a tumorigenic line of chinese hamster fibroblasts as well as serum-incubated human fibroblasts, show increased expression of GRO (36). In addition, some immortalized fibroblast cell lines are known to be responsive to MGSA/GRO, indicating that this cytokine, which originally has been detected in supernatants of melanoma cells, acts as a growth hormone for different cells (37). Apart from this, autocrine growth-stimulating activity of MGSA/GRO is well documented (37).

It would be interesting to know whether dermal fibroblasts are one or the major source of NAP such as NAP-1/IL-8 as well as MGSA/GRO in neutrophilic skin diseases such as psoriasis where both cytokines are found to be excessively increased (34). Because keratinocytes as the major cell source apparently are able to produce only low amounts of NAP-1/IL-8 (J.-M. Schröder, H. -H. Henneicke, M. Sticherling, M. Herlin, and E. Christophers, manuscript in preparation) fibroblasts now need to be considered as important producers of NAP-1/IL-8 and MGSA/GRO.

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IL-8 PRODUCTION BY HUMAN POLYMORPHONUCLEAR LEUKOCYTES

The Chemoattractant Formyl-Methionyl-Leucyl-Phenylalanine Induces the Gene Expression and Release of IL-8 through a Pertussis Toxin-Sensitive Pathway[1]

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IL-8 is a novel chemotactic cytokine, produced by a variety of blood and tissue cells, that has marked activating effects on polymorphonuclear leukocytes (PMN). We report that IL-8 is produced and released by human PMN after stimulation with the chemotactic agonist FMLP. Release of IL-8 in response to FMLP was transient and not influenced by PMN adherence or by the absence of serum in the medium. Maximum yields were usually obtained with 10 nM FMLP within 2 h of stimulation (0.5–3.5 ng/ml/7·10⁶ cells, range of 17 different donors). IL-8 release was dependent on FMLP-induced de novo protein synthesis because it was inhibited by cycloheximide, was paralleled by enhanced expression of IL-8 mRNA and was potentiated from two- to sixfold after preincubation of PMN with cytochalasin B. The FMLP effect was direct and not dependent on LPS or on contaminating monocytes, which showed only low responsiveness to FMLP. Pretreatment of PMN with pertussis toxin prevented FMLP-dependent IL-8 production, the effect being evident both at the level of mRNA expression and protein secretion. In addition, two other chemoattractans, platelet-activating factor and C5a, were found capable to induce release of IL-8 by PMN. The results of this study suggest that chemotactically stimulated PMN may be able to amplify the recruitment process of PMN to the inflammatory site by releasing IL-8. As a long-lived cytokine, IL-8 could markedly prolong the attractant effect.

IL-8 is a novel chemotactic cytokine with high selectivity for PMN[3] (1). It was originally discovered as a product of LPS-stimulated monocytes (2, 3) and subsequently found to be expressed in a wide variety of tissue cells (1). Production of IL-8 by mononuclear phagocytes can be induced by different stimuli, including cytokines, immune complexes, and phagocytosable particles (4, 5). In contrast, tissue cells generally respond only to IL-1 and TNF (1).

It was recently shown that PMN have the capability to produce IL-8 (5, 6), indicating that these cells can by themselves enhance PMN recruitment, and thus antimicrobial defense through the local production of IL-8 at sites of infection. To further explore this potential amplification mechanism of antimicrobial defense, we have studied the effects of the potent chemotactic agonists FMLP, and other chemoattractants, on IL-8 production by human PMN.

MATERIALS AND METHODS

Cell purification and culture. PMN preparations, virtually free of monocytes, as assessed by Wright staining and nonspecific esterase cytochemistry, were obtained as described (5). Monocytes were purified from PBMC by adherence to the plastic (5). Immediately after purification, PMN (7×10^6/ml, unless indicated) and monocytes (5×10^5/ml) were stimulated with different concentrations of FMLP, PAF, C5a, or LPS (from *Escherichia coli* 026:B6), depending on the experiment. Cells were cultured at 37°C in polystyrene flasks or six-well tissue culture plates (Nunc, Roskilde, Denmark), using RPMI 1640 medium supplemented with antibiotics and 10% low endotoxin FCS (<16 pg/ml, Seromed, Biochrom KG, Berlin). Cell-free supernatants were collected after different times and IL-8 was determined. The viability of stimulated PMN after 20 h in culture was more than 90%, as estimated by trypan blue exclusion. All reagents were purchased from Sigma Chemical Co. (St. Louis, MO), and all solutions were prepared with endotoxin-free water for clinical use (7).

IL-8 assay. Antigenic IL-8 was quantitated in the cell-free culture supernatants, using a double-ligand ELISA method (8). Samples and standard solutions of rIL-8 (0.03–2 ng/ml), both in duplicate, were incubated for 2 h at 37°C in microtiter plates precoated with a mouse anti-IL-8 mAb. After washings, an alkaline phosphatase-conjugated goat anti-IL-8 pAb was added to the plates for 2 more h. Then, the enzyme activity was determined using *p*-nitrophenylphosphate as substrate. Cell-free culture supernatants were usually stored at −70°C before IL-8 determination.

RNA isolation and Northern blot analysis. Total RNA was extracted from samples of 7×10^7 PMN, size separated, blotted onto a nylon membrane (7), and hybridized with a 0.7-kb *Eco*RI fragment, prepared from the cDNA for human IL-8 kindly provided by Dr. I. Lindley (Sandoz, Vienna, Austria) (9). Extent of hybridization was quantitated by laser densitometry of the autoradiographs and was plotted after normalization on the basis of hybridization to phosphoglycerate kinase (PGK) mRNA (7).

O_2^- production. A total of 100 μl of PMN suspensions (15×10^5/ml) was added to each well of tissue culture grade polystyrene 96 well plates (Nunc). After 1 h of incubation at 37°C, 100 μl of HBSS containing 2 mM $CaCl_2$, 10 mM glucose, 2 mM NaN_3, 160 μM cytochrome C, and different concentrations of FMLP were added. The plates were incubated at 37°C in an automated EL34 microplate

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[3] Abbreviations used in this paper: PMN, polymorphonuclear leukocytes; CHX, cycloheximide; CB, cytochalasin B; PAF, platelet-activating factor; PT, pertussis toxin.

reader (Biotec Instruments) and absorbance at 550 and 468 nm was recorded at intervals of 1 min for 5 min. O_2^- production in nmol was calculated using an extinction coefficient of 24.5 mM (10).

Statistical analysis. Data are expressed as means ± SEM. The statistical evaluation of the data was performed by the Student's *t*-test for paired data and considered significant if $p < 0.05$.

RESULTS

Effect of FMLP on IL-8 release by PMN. Figure 1 reports the results of 17 separate experiments demonstrating that PMN exposed to FMLP for 2 h release IL-8. The response of PMN from different donors varied considerably, ranging from 563 to 3464 pg/ml (1578 ± 179, $n = 17$) to 10 nM FMLP, and from 441 to 1061 pg/ml (639 ± 140, $n = 6$) to 1 μM FMLP. Virtually no release of IL-8 was observed under control conditions (40 ± 11 pg/ml, $n = 17$), and intermediate levels (662 ± 146 pg/ml, $n = 6$) were obtained on exposure to concentrations of LPS (1 μg/ml), which were shown to be optimal (M. A. Cassatella, unpublished observations). When the PMN were stimulated with FMLP in rotating 50-ml polypropylene tubes to prevent adherence, or in the absence of serum in appropriately FCS-precoated culture flasks (11, 12), the yield of IL-8 recovered was similar to that obtained from PMN cultured in medium supplemented with FCS. Furthermore, the effect of FMLP on IL-8 release was not affected by polymyxin B, which however, almost completely abolished the effect of LPS, and was potentiated by a previous incubation of PMN with 10 ng/ml LPS for 90 min (data not shown).

Figure 2 (*top*), representing a typical experiment, shows that the effect of FMLP was concentration dependent: the yield of IL-8 was already significant at 1 nM, peaked at 10 nM, and decreased progressively when the stimulus concentration was raised to 100 and 1000 nM. As also shown in Figure 1, statistical significant difference between 10 nM and 1 μM was found ($p < 0.02$). Comparatively higher concentrations of FMLP were necessary for the maximal induction of the respiratory burst. In fact, in agreement with the observations of Korchak et al. (13), O_2^- release was barely detectable at 10 nM and reached a maximum between 100 and 1000 nM (Fig. 2, *bottom*).

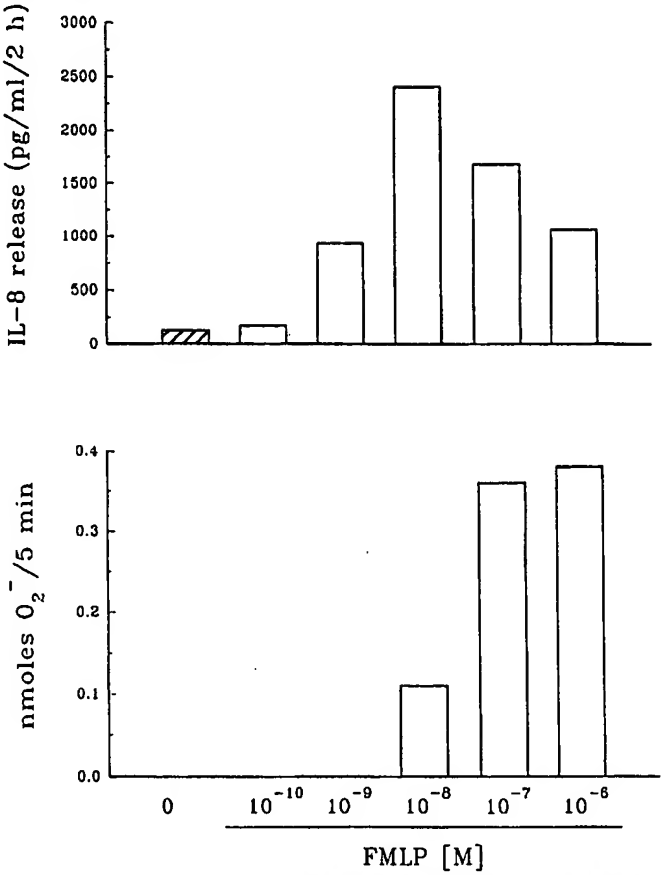


Figure 2. Concentration-dependent effect of FMLP on the release of IL-8 and O_2^- by human PMN. Assays were performed in parallel using PMN from the same donor. These results are representative for four experiments performed under the same conditions.

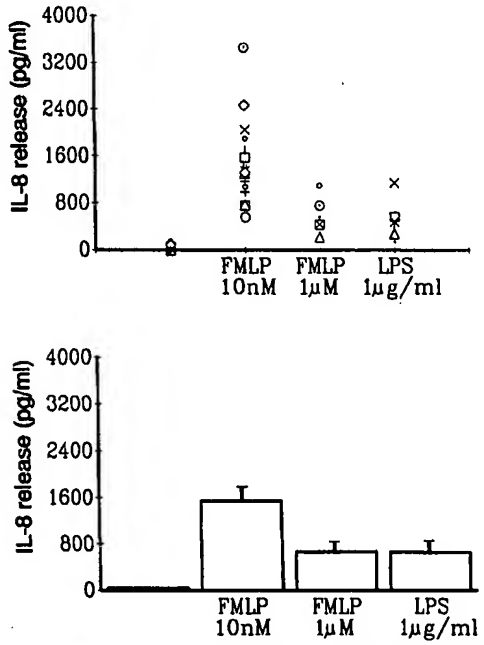


Figure 1. Release of IL-8 from PMN stimulated with different concentrations of FMLP or LPS. PMN (7×10^6/ml) from 17 normal individuals were cultured for 2 h at 37°C in the presence or absence of FMLP 10 nM ($n = 17$), FMLP 1 μM ($n = 6$), and LPS 1 μg/ml ($n = 6$). IL-8 was determined by ELISA in the cell-free supernatants. The figure shows the single mean values of duplicate assays from each experiments (*top*) and the mean values ± SEM for each stimulus (*bottom*). A statistical significant difference between 10 nM and 1 μM FMLP ($p < 0.02$) was observed.

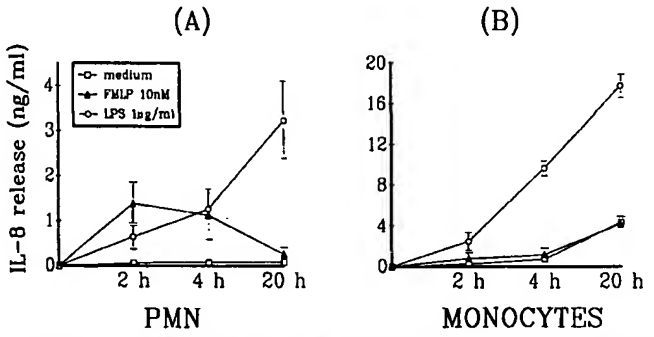


Figure 3. Time course of IL-8 release by human PMN and monocytes stimulated with FMLP or LPS. PMN (*A*) and monocytes (*B*) were stimulated with 10 nM FMLP and 1 μg/ml LPS for the times indicated, and IL-8 was then determined in the cell-free supernatants by ELISA. The figure shows the means ± SEM of duplicate assays for each time point, obtained from six experiments performed under the same conditions.

The time course of stimulus-dependent IL-8 release is shown in Figure 3*A*. In most experiments the highest amounts in response to stimulation with 10 nM FMLP

were recovered after 2 h. The yield of IL-8 was generally lower after 4 h (1129 ± 504 pg/ml, $n = 6$), and continued to decrease after longer times of incubation (260 ± 89 pg/ml/20 h). By contrast, even if after 2 h it was more than 50% less than that of FMLP, the yield of IL-8 upon stimulation with LPS progressively increased up to 20 h, reaching values of 3230 ± 824 pg/ml (Fig. 3*A*). Although considerable, these levels were on average lower than those obtained upon stimulation by phagocytosis (5).

Inasmuch as monocytes are high producers of IL-8, it was necessary to exclude that the effects observed with the PMN preparations could be due to contamination by mononuclear cells. As shown in Figure 3*B*, the responses of monocytes to FMLP and LPS were quite different from those of PMN. Within 2 h, the yield of IL-8 from cells stimulated with LPS was higher (2480 ± 934 pg/ml, $n = 4$) than that obtained with FMLP (761 ± 433 pg/ml, $p < 0.04$ vs control), although control monocytes released 245 ± 107 pg/ml of IL-8. Between 2 and 20 h, IL-8 release by monocytes increased to the same extent in the presence of (4236 ± 368 pg/ml at 20 h), and in the absence of FMLP (4363 ± 286 pg/ml), suggesting that FMLP did not act further as a stimulus. IL-8 production by unstimulated, adherent monocytes was also reported by Kasahara et al. (14). Furthermore, within 4 h, FMLP failed to enhance the expression of IL-8 mRNA in monocytes (data not shown).

Effect of cytochalasin B on FMLP-induced IL-8 release. Table I summarizes the results of five independent experiments in which PMN were pretreated with 5 μg/ml CB before stimulation with 1 μM FMLP. It is evident that CB enhanced the FMLP-dependent IL-8 release by several fold. This potentiation resulted statistically significant ($p < 0.02$).

Effect of CHX on FMLP-induced IL-8 release. The fact that IL-8 was not released before 30 min of stimulation (not shown), suggested that de novo synthesis must precede release. Table II shows that, in three separate experiments, preexposure of PMN to CHX, an inhibitor of protein synthesis, markedly reduced the yield of IL-8 recovered in FMLP-stimulated supernatants.

TABLE I

Effect of cytochalasin B on IL-8 release by human PMN stimulated with FMLP[a]

Addition	IL-8 (pg/ml)				
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
None	57	17	45	40	47
CB	52	20	65	50	82
FMLP	226	214	822	441	855
CB + FMLP	1260	955	2818	977	1780
Fold enhancement by CB[b]	6.3	4.7	3.5	2.3	2.1

[a] PMN (7×10^6/ml) were pretreated for 5 min with or without 5 μg/ml CB and then stimulated for 2 h with 1 μM FMLP. Mean values of duplicate assays from single experiments performed with different neutrophil preparations.
[b] Calculated after deduction of the respective control values.

TABLE II

Effect of cycloheximide on IL-8 release by human PMN stimulated with FMLP[a]

Addition	IL-8 (pg/ml)		
	Expt. 1	Expt. 2	Expt. 3
None	0	125	86
CHX (20 μg/ml)	0	115	4
FMLP 10 nM	1710	2406	998
CHX + FMLP	30	998	110
% inhibition by CHX[b]	98	63	88

[a] PMN (7×10^6/ml) were pretreated for 15 min with or without CHX and then stimulated for 2 h with FMLP. Mean values of duplicate assays from single experiments performed with different neutrophil preparations.
[b] Calculated after deduction of the respective control values.

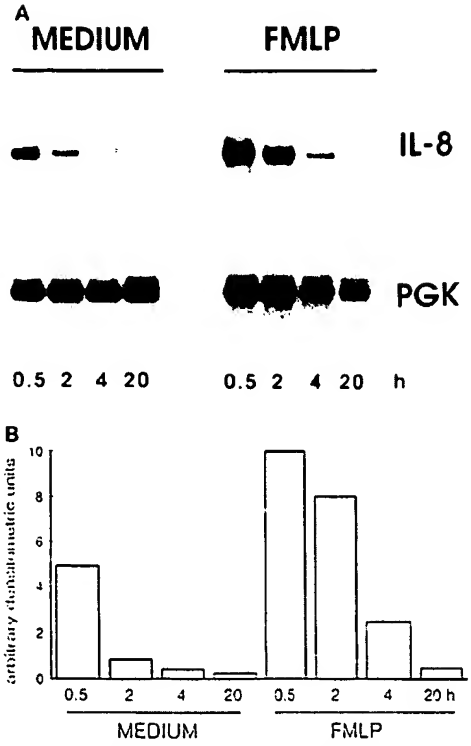


Figure 4. Time course of IL-8 mRNA expression in human PMN stimulated with FMLP. *A*, Samples of 7×10^7 PMN were cultured in the presence or absence of 10 nM FMLP. At the times indicated total RNA was extracted from the cells and Northern blot analysis for IL-8 and PGK transcripts was performed. Ten μg total RNA were loaded per each gel lane. *B*, Quantitative representation of the IL-8 signal. The values on the histograms are plotted as arbitrary densitometric units after normalization on the basis of hybridization to PGK mRNA. The results are representative for three experiments performed under the same conditions.

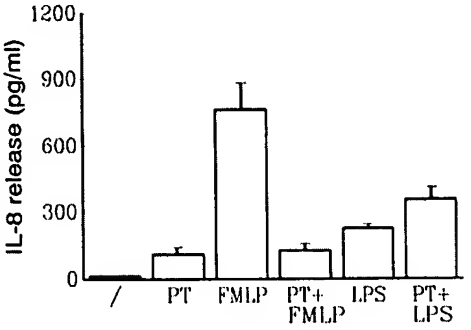
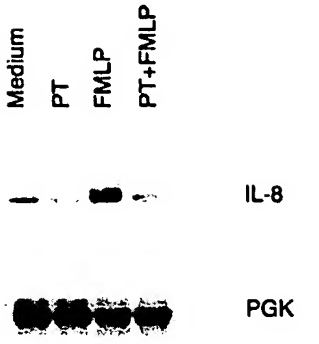


Figure 5. Effect of PT on IL-8 release by PMN stimulated with FMLP or LPS. PMN (5×10^6/ml) were pretreated for 90 min with 500 ng/ml PT before stimulation with 10 nM FMLP or 10 ng/ml LPS. After 90 min cell-free supernatants were collected and IL-8 was determined by ELISA. The results show the means ± SEM of duplicate assays from four experiments performed under similar conditions.

Effect of FMLP on IL-8 mRNA levels in PMN. Figure 4 shows the expression of IL-8 mRNA steady state levels in PMN cultured for different times in the presence or absence of 10 nM FMLP. As previously reported, control PMN constitutively express small amounts of IL-8 mRNA (5) that progressively decrease toward undetectable levels (Fig. 4). In the presence of FMLP, IL-8 mRNA transcripts



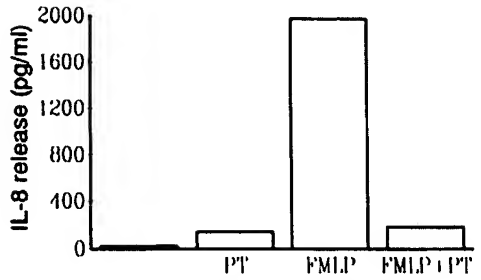


Figure 6. Inhibition of IL-8 mRNA from PMN by PT when given before FMLP stimulation. PMN were pretreated for 90 min with 500 ng/ml PT before stimulation with 10 nM FMLP. After 90 min total RNA was extracted and Northern blot analysis for IL-8 and PGK transcripts performed. Ten μg total RNA were loaded per each gel lane. The effect of PT on the IL-8 release by PMN stimulated with FMLP in the same experiment is shown on the bottom of the figure. One more Northern blot was performed and gave identical results.

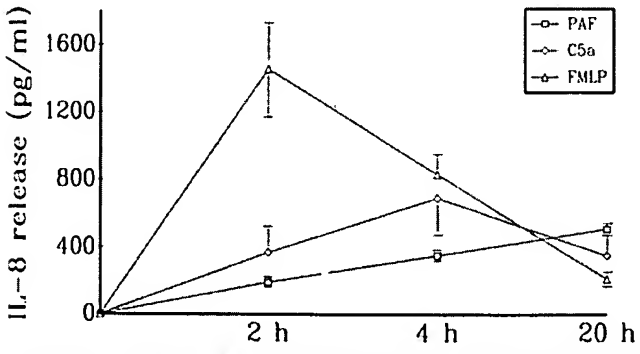


Figure 7. Time course of IL-8 release by human PMN stimulated with PAF and C5a. PMN (5×10^6/ml) were cultured for the times indicated in the presence of 1 μM PAF and 100 ng/ml C5a. IL-8 was determined in the supernatants by ELISA. The figure shows the means ± SEM of duplicate assays for each time point, obtained from three experiments performed under the same conditions.

increased by 30 min and more markedly at 2 h; then they declined to almost disappear over the next 18 h.

Effect of PT. Inasmuch as the neutrophil responses induced by FMLP, such as exocytosis and the respiratory burst, are mediated by a PT-inhibitable signal transduction process, the effect of pretreatment of the PMN with PT on the yield of IL-8 was tested. As shown in Figure 5, which summarizes the results of four experiments, a pretreatment of PMN (5×10^6/ml) with 500 ng/ml PT before stimulation with 10 nM FMLP for 90 min decreased FMLP-induced IL-8 production to control levels, but did not affect the production in response to LPS. Interestingly, PT alone enhanced slightly the yield of IL-8 (110 ± 47 pg/ml). Northern blot analysis demonstrated that PT also prevented the FMLP-dependent enhancement of IL-8 mRNA expression (Fig. 6). In parallel we also tested the effect PT on the respiratory burst. O_2^- generation in response to FMLP was prevented, confirming our previous results (15), although the respiratory burst induced by PMA remained unaffected, indicating that the action of PT was selective and did not cause appreciable toxicity.

Effect of PAF and C5a on IL-8 release by PMN. To determine if production of IL-8 could be reproduced with other chemotactic factors, PMN were incubated for various times with C5a and PAF, at concentrations which preliminary experiments indicated to be the more effective (100 ng/ml for C5a, and 1 μM for PAF). Figure 7 shows that, although at lower extent compared to FMLP, both C5a and PAF were able to induce a significant release of IL-8, already after 2 h (192 ± 20 pg/ml $p < 0.05$ for PAF, and 370 ± 172 pg/ml $p < 0.04$ for C5a, $n = 3$). Interestingly, IL-8 secretion induced by PAF increased during the time of incubation, whereas that induced by C5a followed the same kinetics of FMLP.

DISCUSSION

Our study demonstrates that PMN produce and release IL-8 in response to stimulation with the chemotactic agonist FMLP. The release of IL-8 was strictly dependent on stimulation, occurred at FMLP concentrations that are optimal for chemotaxis but not for respiratory burst and secretion (16), and was not appreciably influenced by the absence of serum in the medium or by conditions preventing PMN adherence to the plastic. Interestingly, FMLP did not induce a significant release of IL-8 by monocytes, cells that otherwise respond to a wide variety of stimuli including LPS, cytokines, immune complexes, and phagocytosis (4, 5). These observations suggest that chemotaxis-dependent IL-8 production may be restricted to PMN.

FMLP appears to act transiently on PMN as compared with LPS or phagocytosis (5). Significant amounts of IL-8 were detected in the supernatants after 1 to 2 h and release decreased thereafter. The release, however, was preceded by a lag of 20 to 30 min, and was, therefore, considerably delayed as compared with the granule exocytosis, a typical response to FMLP that is completed within a few min (17, 18). IL-8 release depended on de novo protein synthesis, because it was almost completely prevented by CHX and was preceded by an enhancement of the IL-8 mRNA levels. Presence of specific IL-8 mRNA was also detected in most samples of untreated PMN (5), but in the absence of stimulation the level of these transcripts decreased almost completely within a few hours of cell culture. Expression of IL-8 mRNA in untreated cells was not caused by adherence to plastic (14), because usually freshly isolated PMN were found to possess IL-8 but not TNF transcripts (M. A. Cassatella, unpublished observations). This constitutive IL-8 mRNA could result from mechanical stress during cell preparation, or could represent a constitutive RNA pool that may facilitate the rapid appearance of the mature protein when the PMN are stimulated. Despite the presence of specific RNA, no secretion of immunoreactive IL-8 was observed in any of more than 20 different control preparations tested. The

transient secretion of IL-8 and the fact that the yield was usually higher after stimulation with 10 rather than 100 or 1000 nM FMLP are difficult to explain. It is conceivable that IL-8 is degraded by proteases and/or oxygen-derived radicals that are released predominantly at higher concentrations, or that high doses of FMLP initiate signal transduction events with inhibitory effects. As already observed in the case of many other functions triggered by the chemoattractant (19), of particular significance is the fact that pretreatment of the PMN with PT fully prevented the synthesis and consequent release of IL-8. This suggests that the G_i-type GTP-binding proteins that couple FMLP-bound receptors to effector systems involved in shape change, exocytosis, and the activation of the respiratory burst also control gene regulation.

The fact that a chemotactic peptide agonist is able to induce long term changes in the neutrophils, in addition to the typical and rapid motor and granule release responses, is remarkable. The inductive effect of FMLP resulting in IL-8 release was strongly enhanced by pretreatment of the cells with CB. This fungal metabolite blocks the contractile system (20) and also enhances agonist-dependent granule exocytosis and respiratory burst (17). Thus, it would appear that similar molecular mechanisms control the immediate and the inductive responses to FMLP, but further studies are necessary to elucidate whether CB acts pretraslationally or simply favors IL-8 secretion.

In a few experiments we searched for a possible effect of IL-8 on its own expression. By Northern blot analysis we found significantly enhanced levels of IL-8 transcripts in PMN that had been exposed to IL-8 for times longer than 1 h (not shown). Release could not be tested in this case because of the presence of IL-8 as a stimulus. However, we found that also other chemotactic factors such as PAF and C5a were able to determine IL-8 release by human PMN.

The results of our study suggest that chemotactically stimulated neutrophils have the capability to elaborate chemoattractant peptides that could amplify the recruitment process. The principle of amplification is typical for defense responses. IL-8 is very long-lived compared to other chemotactic agonists including FMLP (21), and its generation by FMLP-stimulated PMN could result in a marked prolongation of the attractant effect. Activated PMN produce PAF and leukotriene B_4, which are both chemoattractants and thus potential amplifiers of the response (22). In contrast to IL-8, however, these bioactive lipids are rapidly inactivated and thus presumably short-acting.

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In vivo results of p43 regarding induction of macrophage production

Skin Wound Generation and Immunofluorescence Staining

For the *in vivo* wound experiments, we used 8-week old male C57BL6 mice ($p43^{+/+}$ and $p43^{-/-}$). We anesthetized the mice with an intra-peritoneal injection of 2.5% avertin (100□l/10g), shaved the dorsum, and disinfected the skin with 70% alcohol. A 0.5cm diameter circle was marked on the skin of the mid-dorsal region, and full-thickness excisional wounds, including the skin and panniculus carnosus muscle, were created using scissors. The wounds were left uncovered without a dressing. One wound was generated per mouse. We fixed the isolated wounds with 4% paraformaldehyde at 4°C overnight. The tissues were washed with PBS, incubated in 30% sucrose for 4h, and finally frozen at –70 °C in optimal cutting temperature (OCT) compound. The frozen sections (6□m) were attached to silane-coated slides, treated with PBS, blocked with PBS containing 0.1% Tween 20 and 1% skim milk, and reacted with antibodies specific to p43, MOMA-2 (Serotec), and Ki67 (Santa Cruz Biotechnology) at 37 °C for 2h. We washed the slides with PBS containing 0.1% Tween 20 and incubated them at 37°C for 1h with the FITC-conjugated secondary antibody. The nuclei were counterstained with propidium iodide (10□g/ml) for 10min, and the sections were examined under the confocal immunofluorescence microscopy (□-Radiance, BioRad).

Figure. Immunofluorescence staining of macrophages in the wound region.

A 0.5 cm diameter full-thickness portion of the dorsal skin was removed and cross sections of the wound area were reacted with anti-MOMA2 antibody and stained with FITC-conjugated secondary antibody.